PD1-positive tumor-infiltrating lymphocytes are associated with poor clinical outcome after pulmonary metastasectomy for colorectal cancer

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Abstract: Pulmonary metastasectomy (PM) is routinely performed in colorectal cancer (CRC) patients with oligometastatic spreading to the lungs. Patients with an aggressive tumor phenotype should be excluded from PM, since its benefit is outweighed by early tumor recurrence and impaired prognosis. Expression of PD-1 and its ligands are prognostic factors in a variety of primary tumors. However, their impact on patients' outcome in the setting of PM for CRC has not been evaluated before. 53 CRC patients with pulmonary metastases receiving PM with curative intent were included in this study. Tissue samples of resected pulmonary metastases and available corresponding primary tumors were collected and assessed for PD-1, PD-L1 and PD-L2 expression by tumor-infiltrating lymphocytes (TILs) and tumor cells. Expression patterns were correlated with clinical outcome parameters. PD-1 and PD-L1 expression was commonly found in TILs and tumor cells. Expression levels significantly differed between metastases and primary tumors. High PD-1 expression by TILs was associated with impaired overall survival (low vs high expression (mean, 95% CI): 78 mo (60-96) vs 35 mo (25-44); p = 0.011). Additionally, the subgroup of patients, who experienced an upgrading in their TILs/PD1 status between primary and metastasis had a worse survival outcome compared with patients with the same grade or a downgrading (34 mo (26-42) vs 96 mo (72-120); p = 0.004). Thus, PD-1 expression by TILs is a strong prognostic marker in CRC patients with pulmonary spreading treated by PM. Moreover, this study provides a rationale for a therapeutic PD-1 pathway blockade in the treatment of CRC lung metastases. Future, large-scale studies are warranted to validate the findings of this single-center, retrospective analysis.

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PD1-positive tumor-infiltrating lymphocytes are associated with poor clinical outcome after pulmonary metastasectomy for CRC

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Abstract

Background

Pulmonary metastasectomy (PM) is routinely performed in colorectal cancer (CRC) patients with oligometastatic spreading to the lungs. Patients with an aggressive tumor phenotype should be excluded from PM, since its benefit is outweighed by early tumor recurrence and impaired prognosis. Expression of PD-1 and its ligands are prognostic factors in a variety of primary tumors. However, their impact on patients' outcome in the setting of PM for CRC has not been evaluated before.

Material and Methods

53 CRC patients with pulmonary metastases receiving PM with curative intent between April 2009 and November 2013 were included in this study. Tissue samples of resected pulmonary metastases and available corresponding primary tumors were collected and assessed by immunohistochemistry for PD-1, PD-L1 and PD-L2 expression by tumor-infiltrating lymphocytes (TILs) and tumor cells. Results were correlated with clinical outcome parameters.

Results

PD-1 and PD-L1 expression was commonly found in TILs and tumor cells. Expression levels were significantly different between metastases and primary tumors. High PD-1 expression by TILs was associated with impaired overall survival (low vs high expression (mean, 95% CI): 78 months (60-96) vs 35 months (25-44); p=0.011). Additionally, the subgroup of patients, who experienced an upgrading in their TILs/PD1 status between primary and metastasis had a worse survival outcome compared to patients with the same grade or a downgrading (34 months (26-42) vs 108 months (87-129); p<0.001).

Conclusions

PD-1 expression by TILs is a strong prognostic marker in CRC patients with pulmonary spreading treated by PM. Moreover, this study provides a rationale for a therapeutic PD-1 pathway blockade in the treatment of CRC lung metastases. Further studies are warranted to confirm these findings in a larger study cohort

Introduction

Pulmonary metastasectomy (PM) is one of the corner stones in the treatment of oligometastasized colorectal cancer (CRC). By removing all gross tumor spreading to the lungs, 5-year survival rates of up to 50-70% can be achieved.[1, 2] This is in strong contrast to survival rates of patients, who do not qualify for a surgical treatment, with only 1 out of 10 patients being alive at 5 years.[3] One of the main problems in PM is the selection of patients for the procedure. Most thoracic surgeons are reluctant to offer PM to patients with an aggressive tumor phenotype. In these cases, a removal of pulmonary metastases is frequently challenged by an early tumor recurrence, thus exposing patients mainly to the risks of an operation without a clear oncological benefit. To date there is a lack of clear selection criteria for PM in patients with CRC. Recently, markers that determine tumor behavior and aggressiveness have been in the spotlight of research.[4, 5]

Programmed death-1 (PD-1) and its ligands PD-L1 and PD-L2 are important immune checkpoints. PD-1 is an inhibitory co-signal on activated lymphocytes and plays a crucial role in regulating the magnitude and quality of T-cell responses. Immunogenic tumors can escape immune surveillance by an upregulation of PD-1 ligands and thus leading to an inactivation of the endogenous anti-tumor immune defense. Blocking of the PD-1 pathway by therapeutic antibodies is a novel targeted therapy, which counteracts PD-1 dependent tumor immune escape mechanisms. In clinical trials, PD-1 inhibitors have been proven effective in patients with advanced melanoma, renal cell carcinoma, non-small-cell lung cancer, and colorectal carcinoma.[6]

A prognostic impact of PD-1 and PD-L1 expression has been described for several tumor entities e.g. breast cancer, hepatocellular carcinoma and esophageal cancer.[7 8 9] However, in primary CRC PD-1 and PD-L1 expression are is paradoxically associated with favorable outcome parameters. PD-1 and PD-L1 expressing tumor-infiltrating lymphocytes (TIL) at the primary tumor site lead to prolonged recurrence free and overall survival.[10, 11] This unique finding in CRC has been controversially discussed in the literature and is explained by the gut specific microenvironment and particular features of the intestinal immune system.

To the best of our knowledge the expression of PD-1 and its ligands has not been described in CRC lung metastases. Furthermore, its prognostic impact on outcome parameters in patients receiving curative PM has not been evaluated before.

Material and Methods

Study population

53 CRC patients with pulmonary metastases receiving PM with curative intent between April 2009 and November 2013 were included in this single-center study. In the case a PM has been performed before the inclusion period, the specimen of the first PM was also assessed. For 31 (58%) patients paraffin embedded specimens from the primary tumor were available. Tumor staging prior to metastasectomy was performed by abdominal and thoracic computed tomography (CT) scan. In case of an inconclusive CT, positron emission tomography (PET) was added to exclude extrathoracic spreading. All patients were operated through a muscle-sparing anterolateral or posterior incision. Lungs were bimanually palpated for occult lesions and a lymph nodes sampling was performed. Complete resection (R0) was achieved in all patients. Patients were postoperatively followed-up in three-months intervals with chest and abdominal CT scans during the first year and were seen every six months thereafter.

Lung metastasis free survival (LMFS) was defined as the time between the diagnosis of the primary tumor and the diagnosis of metastatic spread to the lungs. Time to recurrence represented the time between PM and the first evidence of metastatic recurrence at any site. Overall survival (OS) was defined as the period of time between pulmonary metastasectomy and death of any cause.

This study was approved by the ethics committee of the Medical University of Vienna (EK#: 1097/2014) and was performed according to the Declaration of Helsinki and the Good Scientific Practice guidelines of the Medical University of Vienna.

Immunohistochemistry

3-5µm thick sections were deparaffinized and rehydrated in graded series: X-TRA-Solv 8 (Medite, # 41-5212-00) - 15 min at 68°C; Xylol – 5 min room temperature (RT), 100% EtOH - 5 min RT; 96% EtOH - 5 min RT; 80% EtOH - 5 min RT; distilled water - 2 min RT. Antigen retrieval with target retrieval solution (pH9) was performed. For this purpose the slides were heated to 115ºC for 10 min in Dako Cytomation Pascal Pressure Cooker. After cooling to RT and washing with TBS buffer, 3% hydrogen peroxide in distilled water was used to block the endogenous peroxidase activity (10 min, RT). The following antibodies were used for PD stains: anti-human PD1 antibody (R&D Systems, # AF 1086, dilution 1:20), PD-L1 (Cell Signaling, clone: E1L3N, dilution: 1:25), PD-L2 (Cell Signaling, clone: D7U8C, dilution: 1:25). The slides were automatically processed with a Dako Autostainer Plus System. Visualization was implemented with streptavidin conjugated to alkaline phosphatase. Cell nuclei were counterstained by Mayer's hematoxylin.

For each slide, four different areas of tumor tissue were selected for analysis. Tumor cells and tumor-infiltrating lymphocytes in these areas were independently evaluated by two investigators blinded to the clinical data. If the rating differed, the slide was re-discussed and a consensus was found using a multi-head microscope. Slides were examined at ×400 magnification, and the staining rate (percentage of tumor cells and lymphocytes showing positive staining) was determined. PD-1, PD-L1 and PD-L2 expression was categorized into 0: no positive cells, 1+: 5-25% of cells, 2+: 26-50% of cells, 3+: 51-75% of cells and 4+: 76-100% of cells. For some analysis a dichotomization was used. 0-50% positive PD-1 cells were defined as PD-1^{low}, 51-100% positive PD-1 cells as PD-1^{high}.

Immunostaining for CD3 (clone SP7, #RM9107-S1, Thermo Fisher Scientific, Cheshire, UK), CD8 (clone C8/144B, #M7103, Dako, Glostrup, Denmark), CD45RO (clone UCHL1, #M074201, Dako, Glostrup, Denmark) were performed using an autostainer (Benchmark Ultra, Ventana Medical Systems, Tuscon, USA), as previously described.[12] In negative controls the primary antibody was omitted. A

mediastinal lymph node served as positive control. As previously published, tumor-infiltrating lymphocytes were classified semi-quantitatively by two independent observers.[12] The following score for TIL was used: scattered (+), intermediate (2+), dense infiltrate (3+), very dense infiltrate (4+). Grades of none (-), sparse (1+), intermediate (2+), high (3+) were used to classify the proportion of CD8+ or CD45RO+ cells in the immune infiltrate.

Statistical analysis

All data were evaluated using SPSS 23 (SPSS Inc., Chicago, USA). Nominal variables were compared using Fisher's exact test and chi-square test. Correlations were calculated using the Kendall-Tau equation. Survival curves were estimated by Kaplan-Meier plots and the differences between the groups were compared using the log-rank test. All performed tests were two-sided. P-values < 0.05 were considered statistically significant. Due to the hypothesis generating approach of the study no correction for multiple testing was applied.[13]

Results

Patients' characteristics

A total of 53 (30 male and 23 female) patients were included in the study. The primary tumor site was colon for 57% and rectum for 43% of patients. Most patients were in UICC stage III and IV at the time of prognosis with a high number of already N positive tumors. Sixteen patients had a previous liver metastasectomy before they were operated for their pulmonary nodules. The majority of patients (76%) presented with a singular lung metastasis. Uniformingly, a high number had received chemotherapeutic treatment(s) before metastasectomy. 40 out of 53 patients were treated with pseudo-adjuvant chemotherapy after resection of pulmonary metastases. None of the patients received a PD-1 blocking agent during the study period. The median follow-up after metastasectomy was 35 months (range 4-137). Patients' characteristics are summarized in Table 1.

Characterization of the immune-infiltrate

A lymphocytic immune infiltrate was seen in nearly all metastases with a broad distribution ranging from scattered to very dense infiltrates. In most patients the immune infiltrate consisted of a high proportion of CD8+ and CD45RO+ lymphocytes. A detailed description of tumor-infiltrating lymphocytes is presented in Table 2.

Density and distribution of PD-1, PD-L1 and PD-L2 in pulmonary metastases

Quality of Immunohistochemical stainings was sufficient in 52/53 (98.1%) for PD-1, 51/53 (96.2%) for PD-L1 and 52/53 (98.1%) for PD-L2 stainings, respectively. A detailed list of the density of PD-1 and its ligands in TILs and tumor cells is shown in Table 2 and representative images are provided in Figure 1. A high number of TILs were positive for PD-1. Although staining intensity was generally lower in tumor cells, PD-1 expression was also a commonly found feature on malignant cells. PD-L1 was frequently expressed by TILs as well as tumor cells. However, PD-L2 expressing tumor cells were only found in three patients and TILs were all negative for PD-L2. There was no association of PD-1 and PD-L1 expression on tumor cells and TILs with clinicopathological characteristics of the patients (Table 3).

Correlation of PD-1 and PD-L1 expression in pulmonary metastases and corresponding primary CRC

The pattern of PD-1 and PD-L1 expression was distinct between pulmonary metastases and their corresponding primaries (Figure 2). There was an upgrading in TILs/PD-1 expression in 14 patients (52%), seven patients (26%) had a similar expression and six patients (22%) had a lower PD-1 expression in their metastasis compared to the primary tumor. Contrarily, the expression of PD-L1 in TILs was less variable between primary tumor and metastasis. Fifteen patients (57%) were classified the same grade, but only four and seven patients were upgraded and downgraded, respectively. Changes between PD-L1 expression of TILs and tumor cells between primary tumors and metastases are summarized in Suppl. Table 1.

Impact of PD-1 and PD-L1 expression on outcome parameters

Univariate outcome analyses of recurrence-free survival and overall survival after pulmonary metastasectomy were calculated by log-rank tests. Neither the expression of PD-1 nor its ligands by TILs were prognostic for time to recurrence (Figure 3A, Figure 4A). However, PD-1 expression on TILs was associated with impaired overall survival (low vs high (mean, 95% CI): 78 months (60-96) vs 35 months (25-44); p=0.011; Figure 3B, Table 4). Additionally, the subgroup of patients, who

experienced an upgrading in their TILs/PD1 status had a worse survival outcome compared to patients with the same grade or a downgrading (34 months (26-42) vs 108 months (87-129); p<0.001, Figure 3C). The prognostic impact of PD-L1 expression on TILs was less obvious. There was a trend towards impaired survival in patients with TILs/PD-L1 upregulation compared to patients with the same or less PD-L1 positive TILs (29 months (22-36) vs 84 months (63-106); p=0.119, Figure 4C, Table 4). Expression of PD-1 and PD-L1 on tumor cells was not associated with outcome parameters (Table 4).

Discussion

The aim of this study was to evaluate the expression of PD-1 and its ligands PD-L1 and PD-L2 in pulmonary metastases from CRC and to correlate the expression pattern with clinical outcome parameters. We found that PD-1 and PD-L1 was highly abundant in TILs in pulmonary nodules as well as in corresponding primary tumors, whereas PD-L2 was only found sporadically. Interestingly, there was a significant heterogeneity of PD-1 and PD-L1 expression between pulmonary metastases and corresponding primaries. High PD-1 expression of TILs in pulmonary metastases was a predictor of impaired survival, with the worst prognosis in patients who had an upgrading in their TILs/PD-1 status between primary and metastasis. PD-L1 expression did not impact overall survival, however, patients with an upgrading of their TILs/PD-L1 showed a trend towards worse outcome.

To the best of our knowledge this is the first structured evaluation of PD-1 expression and its ligands in pulmonary metastases from CRC. The PD1/PD-L1 axis is considered an essential immune checkpoint. PD-1 is primarily expressed by activated lymphocytes and upon triggering by its ligands (PD-L1 and PD-L2) it can represses Th1 cytotoxic immune responses.[14]
Tumor-infiltrating lymphocytes are an important endogenous defense mechanism against cancer. High levels of TILs are associated with a favorable prognosis in various malignancies including lung, kidney, breast and colorectal cancer.[15-18] Especially in CRC CD3+, CD8+ and CD45RO+ TILs have been extensively studied and recently an international consortium was founded to implement a novel staging system including the immune infiltrate ("Immunoscore").[19] High expression of PD-L1 in the tumor microenvironment can lead to an escape from this tumor-specific T-cell immunity. Consequently, therapeutic PD-1 blocking antibodies have been developed to counteract this phenomenon. In clinical trials, PD-1 inhibitors were successfully tested in patients with advanced melanoma, renal cell carcinoma, non-small-cell lung cancer, as well as subsets of colorectal carcinoma.[6] This concept of TILs re-sensibilization by PD-1 pathway blockers has recently been challenged by the observation that anti-PD-1 cancer therapies are also effective in low immunogenic tumors. An explantation for this is the fact, that PD-1 is also expressed by cancer cells, although to a lesser extent. Engaging this cancer intrinsic PD-1 results in increased tumor growth and is downstream mediated by the mTOR pathway.[20 21] Based on these findings, we also evaluated PD-1 and PD-L1 on cancer cells. Both proteins were present in a high percentage of CRC tumor cells. Despite this fact, expression levels did not correlate with clinical outcome parameters (Table 4). Nevertheless, the high number of PD-1 and PD-L1 positive tumor cells in CRC lung metastasis provides a further rationale for a future application of therapeutic PD-1 blockage in the setting of pulmonary spreading.

PD-L1 can be expressed by a variety of cell types, including lymphocytes, endothelial and epithelial cells. Contrarily, PD-L2 expression is limited to antigen presenting cells and macrophages.[22] PD-L2 has also been shown to be expressed by some solid cancers as endometrium cancer and hepatocellular cancer.[23, 24] In our patients we did not find PD-L2 positive cells in the lymphocytic infiltrate and only insignificant expression on tumor cells. We therefore, excuded PD-L2 from further analysis, since its impact on tumor biology and immunosurveillance can be considered minimal.

Linking the number of TILs with their PD-1/PD-L1 expression status is an aspect, which has not been addressed sufficiently in previous studies. The impact of PD-1/PD-L1 axis on immunogenic tumors should theoretically correlate with the density of the immune infiltrate. Despite the fact, that our study population was small, patients with a high number of CD8+ TILs but low expression of PD-1 had the best prognosis with a mean survival of 64 months (48-79). Patients with only few CD8+ cells and expressing PD-1 had the worst prognosis of only 28 months (22-34). Patients with low CD8+ numbers

but low expression of PD-1 accordingly showed intermediated outcome of a mean survival time of 41 months (24-59).

It is commonly accepted within the thoracic surgical and oncological community that patients, who have a high likelihood of early recurrence, should not undergo PM. Despite this general agreement, there is a lack of knowledge on prognostic factors for this group of patients. The indication for PM is still based on clinical features, which have been proposed in the 1970s.[4] The disease free interval between primary and pulmonary metastasis, the number of pulmonary nodules and available alternative treatment regimens are mostly used selection criteria. Recently, several attempts have been made to link markers of tumor biology with patients' outcome. We have previously shown that the immune cellular infiltrate as well as markers of inflammation are strongly associated with recurrence free and overall survival of PM for CRC.[12, 25] This study extends these findings by showing that the PD-1 pathway is a valid prognostic factor in those patients. Additionally, it provides a rationale for the therapeutic feasibility of PD-1 blockage in this subset of CRC patients.

The prognostic role of PD-1 and PD-L1 expression in primary CRC is still a matter of discussion.[10, 26] Unlike most tumors, in which PD-1 overexpression clearly correlates with impaired survival, PD-1 expressions seems to be paradoxically associated with improved outcome in primary CRC.[10, 11] This is possibly based on specific features of the intestine immune system, which is constantly exposed to commensal microbiota. Furthermore, expression of PD-L1 in primary rectal cancer has been shown to be rare or even absent.[27] A similar paradox is the fact that in primary CRC regulatory T-cells (Tregs) are associated with improved survival, whereas they are a poor prognostic factor in most other solid tumors.[28] The local immune response in the lung seems to be profoundly different from the intestine immune system and PD-1/PD-L1 expression of lung cancer infiltrating TILs is associated with poor clinical outcome.[29] This spatial effect on PD-L1 expression has recently been highlighted in a study on lung cancer patients with brain metastasis. Dong and colleagues found a significant difference between different cancer sites and concluded that the PD-1/PD-L1 axis is strongly influenced by the tumor surrounding microenvironment.[30]

There are several limitations to this study. First, it is a single-center, retrospective analysis with only a limited number of patients, thus deductions have to be interpreted with caution. We currently recruit patients within an international multi-institutional study protocol in order to confirm the impact of proposed prognostic markers in CRC PM in a larger cohort. Second, a selection bias cannot be excluded. Patients undergoing PM are a highly selected subset of patients, who do not necessarily represent all patients with lung metastases. One strength of this study is that IHC analysis were performed on full slides, rather than using tissue microarrays (TMA).[11, 19] Although TMA facilitate the analysis of a large number of samples, tissue analysis is limited to a small cylinders of less than one mm in diameter.[31] In our study the size of obtained tissue samples was approximately 10-20mm and four different random spots were used for analysis. This technique minimized the possibility of bias due to tumor heterogeneity.

In conclusion, this study shows that PD-1 and PD-L1 are uniformingly expressed in tumor cells and TILs of resected CRC pulmonary metastases. High expression of PD-1 in TILs reflects an aggressive tumor biology with impaired overall survival. Further studies are warranted to confirm these findings in a larger study cohort.

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Figure Legends

Figure 1 shows representative slides of PD-1 and PD-L1 expressing tumor-infiltrating lymphocytes. Patients were dichotomized into $PD1^{low}$ (0-50% positive cells) $PD1^{high}$ (51-100% positive cells).

Figure 2 depicts the correlation of PD-1 and PD-L1 expression between lung metastases and corresponding primary tumors. There was a broad inconsistency within the study population with either up- or downregulation of PD-1 and PD-L1 expression in TILs and tumor cells.

Figure 3. Kaplan-Meier plots of PD-1/TILs on patients' outcome. PD-1 expression on tumor-infiltrating lymphocytes did not impact time to recurrence after pulmonary metastasectomy (A), but was significantly associated with an impaired overall survival (B). Patients with an upgrading of their PD-1/TIL status had the worst prognosis (C).

Figure 4. Kaplan-Meier plots of PD-L1/TILs on patients' outcome. In contrast to PD-1, PD-L1 was only loosely associated with outcome parameters. A non-significant trend towards worse overall survival in the group of PD-L1 high-expressing TILs was observed (B). Additionally, upgrading of PD-L1/TIL resulted in an impaired prognosis (C).

Table 1. Patients' characteristics

Table 2. Detailed list of PD-1, PD-L1 and PD,L2 expression on tumor cells and tumor-infiltrating lymphocytes in pulmonary metastases and corresponding primary tumors.

Table 3. Association of PD-1 and PD-L1 expression with clinicopathological characteristics

Table 4. Univariate outcome analysis of recurrence-free survival and overall survival after pulmonary metastasectomy

Suppl. Table 1. Changes in PD-1 and PD-L1 expression between metastasis and corresponding primary

Table 1 - Patients' characteristics

	Total study cohort (n=53)	
	n	%
Median age at surgery (range)	64 (33-79)	
Median follow-up after metastasectomy in months (range)	35 (4-137)	
Sex		
Male	30	56.6
Female	23	43.4
Localization of primary tumor		
Colon	30	56.6
Rectum	23	43.4
T stage		
-1	1	2.0
-2	6	12.0
-3	36	72.0
-4	7	14.0
unknown	3	-
N stage		
-0	21	42.0
-1	14	28.0
-2	15	30.0
unknown	3	-
UICC stage of primary tumor		
I	4	8.0
II	13	26.0
III	24	48.0
IV	9	18.0
unknown	3	-
Previous liver metastasis		
Yes	16	30.2
No	37	69.8
Lung metastasis free survival		
<36 months	32	60.4
36-60 months	8	15.1
60 months	9	17.0
No. of pulmonary metastases		
singular	40	75.5
multiple	13	24.5
Chemotherapy before metastasectomy		
Yes	42	79.2
No	11	20.8
Chemotherapy after metastasectomy		
Yes	40	75.5
No	13	24.5

Suppl. Table 1 - Changes in PD-1 and PD-L1 expression between metastasis and corresp

	PD-1			
	TILs		Tumor cells	
	n	%	n	%
downgrading	6	22.2	11	37.9
same grade	7	25.9	12	41.4
upgrading	14	51.9	6	20.7

onding primary

PD-L1			
TILs		Tumor cells	
n	%	n	%
7	26.9	6	21.4
15	57.7	11	39.3
4	15.4	11	39.3

Table 2 - PD1, PD-L1 and PD-L2 Expression

	Metastasis				Primary tumor			
	TILs		Tumor cells		TILs		Tumor cells	
	n	%	n	%	n	%	n	%
PD-1								
negative	5	9.6	5	9.6	4	13.3	2	6.7
1-25%	15	28.8	5	9.6	17	56.7	4	13.3
16-50%	16	30.8	7	13.5	4	13.3	5	16.7
51-75%	13	25	9	17.3	5	16.7	8	26.7
76-100%	3	5.8	26	50.0	0	0.0	11	36.7
PD-L1								
negative	10	19.6	15	29.4	5	16.7	13	43.3
1-25%	33	64.7	27	52.9	19	63.3	15	50.0
16-50%	8	15.7	5	9.8	6	20.0	1	3.3
51-75%	0	0.0	4	7.8	0	0.0	1	3.3
76-100%	0	0.0	0	0.0	0	0.0	0	0.0
PD-L2								
negative	52	100.0	49	94.2	30	100.0	25	83.3
1-25%	0	0.0	3	5.8	0	0.0	2	6.7
16-50%	0	0.0	0	0.0	0	0.0	3	10.0
51-75%	0	0.0	0	0.0	0	0.0	0	0.0
76-100%	0	0.0	0	0.0	0	0.0	0	0.0

	n	%
CD3		
scattered (+)	6	14.3
intermediate (2+)	14	33.3
dense infiltrate (3+)	16	38.3
very dense infiltrate (4+)	6	14.3
CD8		
none (-)	2	4.7
sparse (+)	21	48.8
intermediate (2+)	11	25.6
high (3+)	9	20.9
CD45RO		
none (-)	4	9.5
sparse (+)	13	31
intermediate (2+)	23	54.8
high (3+)	2	4.8

Table 3 - Association of PD-1 and PD-L1 expression with clinicopathological characteristics

	PD-1						PD-L1					
	Tumor-infiltrating lymphocytes			Tumor cells			Tumor-infiltrating lymphocytes			Tumor cells		
	low	*high*	*p*	*low*	*high*	*p*	*negative*	*positive*	*p*	*negative*	*positive*	*p*
Age at surgery												
<64	19	7	0.548	10	16	0.375	3	23	0.139	6	20	0.311
≥64	17	9		7	19		7	18		9	16	
Sex												
Male	18	11	0.209	8	21	0.378	4	24	0.291	7	21	0.446
Female	18	5		9	14		6	17		8	15	
Localization of primary tumor												
Colon	20	10	0.640	9	21	0.629	2	28	0.005	6	24	0.078
Rectum	16	6		8	14		8	13		9	12	
UICC stage of primary tumor												
I	2	1	0.422	1	2	0.868	1	1	0.735	1	1	0.866
II	9	4		4	9		2	11		3	10	
III	18	6		9	15		5	19		7	17	
IV	4	5		2	7		2	7		3	6	
unknown (n=3)												
Previous liver metastasis												
No	25	11	0.960	10	26	0.257	6	29	0.512	10	25	0.846
Yes	11	5		7	9		4	12		5	11	
Lung metastasis free survival												
<36 months	22	9	0.931	10	21	0.778	6	25	0.816	10	21	0.482
36-60 months	6	2		3	5		2	6		3	5	
60 months	6	3		2	7		1	7		1	7	
No. of pulmonary metastases												
singular	27	12	1.000	12	27	0.609	6	32	0.240	9	29	0.125
multiple	9	4		5	8		4	9		6	7	
Lymphatic vessel invasion												
No	25	7	0.079	12	20	0.350	6	25	0.955	8	23	0.482
Yes	11	9		5	15		4	16		7	13	
Chemotherapy before metastasectomy												
No	6	4	0.482	3	7	0.840	1	9	0.393	2	8	0.466
Yes	30	12		14	28		9	32		13	28	
Chemotherapy after metastasectomy												
No	7	5	0.351	4	8	0.957	2	10	0.769	3	9	0.701
Yes	29	11		13	27		8	31		12	27	

Table 4 - Univariate outcome analysis of recurrence-free survival and overall survival after pulmonary metastasectomy

		Recurrence-free survival Univariate analysis (log-rank)		Overall survival Univariate analysis (log-rank)	
		mean survival (months)	p-value	mean survival (months)	p-value
Sex	Male	17 (9-25)	0.081	67 (49-84)	0.685
	Female	31 (17-33)		66 (44-87)	
Age (years)	< 64 yrs	22 (13-32)	0.857	51 (39-62)	0.714
	≥ 64 yrs	26 (15-38)		69 (49-89)	
Location	Colon	19 (13-25)	0.796	68 (48-88)	0.743
	Rectum	28 (16-40)		53 (41-65)	
UICC stage	I + II	23 (12-35)	0.721	43 (32-55)	0.061
	III + IV	26 (17-36)		87 (66-107)	
Chemotherapy before metastasectomy	Yes	24 (16-33	0.683	70 (53-87)	0.538
	No	26 (10-41)		45 (30-59)	
Chemotherapy after metastasectomy	Yes	22 (15-29)	0.589	59 (46-71)	0.269
	No	29 (10-48)		97 (70-125)	
Previous liver metastasis	Yes	14 (7-21)	0.055	49 (34-63)	0.447
	No	30 (20-40)		70 (52-88)	
Lung metastasis free survival	<36	23 (15-31)	0.952	69 (50-89)	0.931
	>=36	22 (12-32)		55 (41-69)	
Number of metastasis	singular	20 (14-25)	0.448	72 (54-89)	0.551
	multiple	33 (14-53)		56 (28-84)	
PD1 - Tumor infiltrating lymphocytes	low	24 (16-33)	0.766	78 (60-96)	**0.011**
	high	24 (12-35)		35 (25-44)	
PD1 - Tumor cells	low	28 (14-41)	0.918	83 (57-109)	0.425
	high	21 (15-28)		59 (45-74)	
PD-L1 - Tumor infiltrating lymphocytes	negative	15 (6-23)	0.310	38 (29-46)	0.307
	positive	26 (17-35)		73 (55-90)	
PD-L1 - Tumor cells	negative	16 (10-23)	0.538	74 (51-98)	0.628
	positive	26 (17-35)		71 (52-89)	

Figure 1

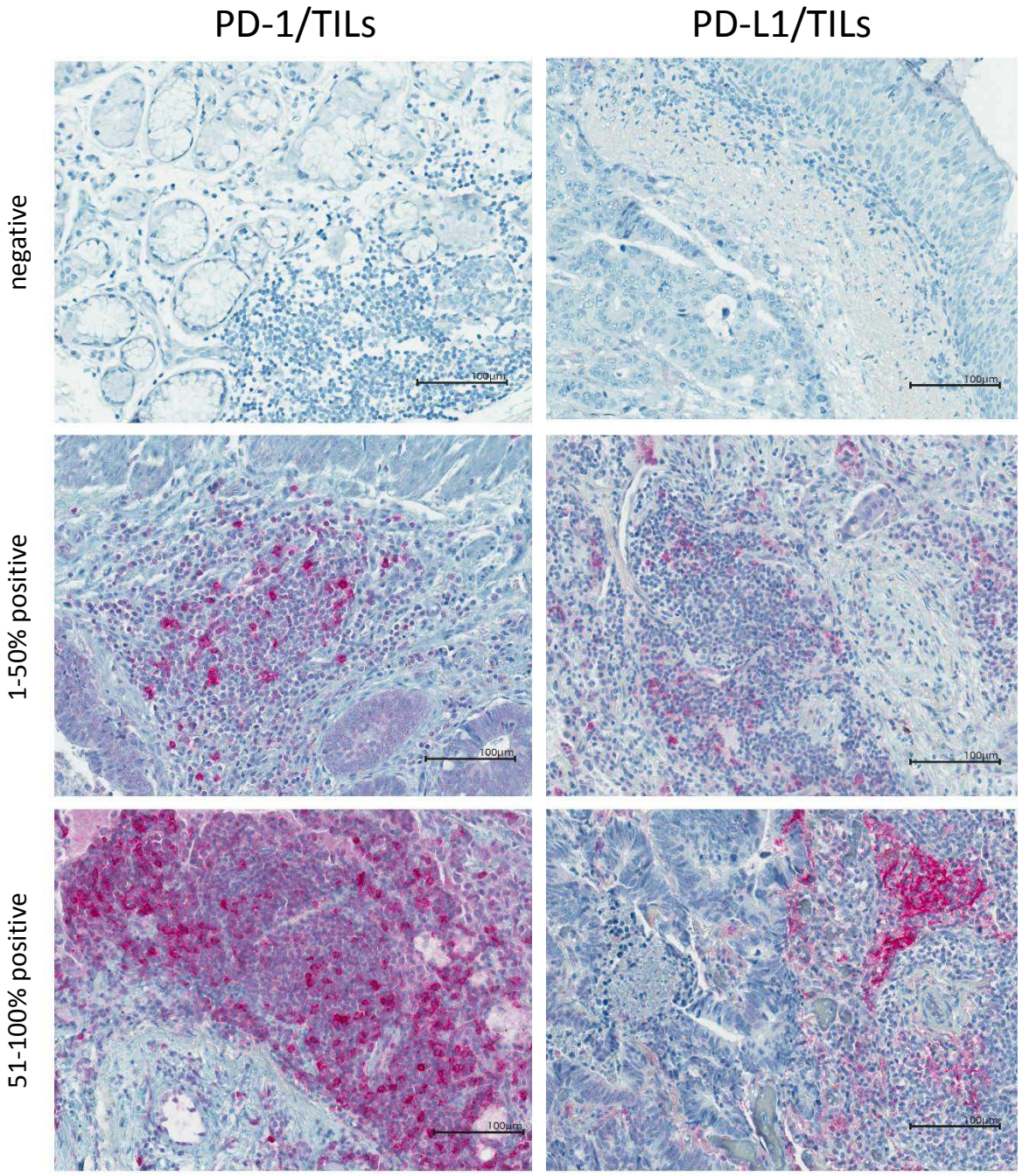
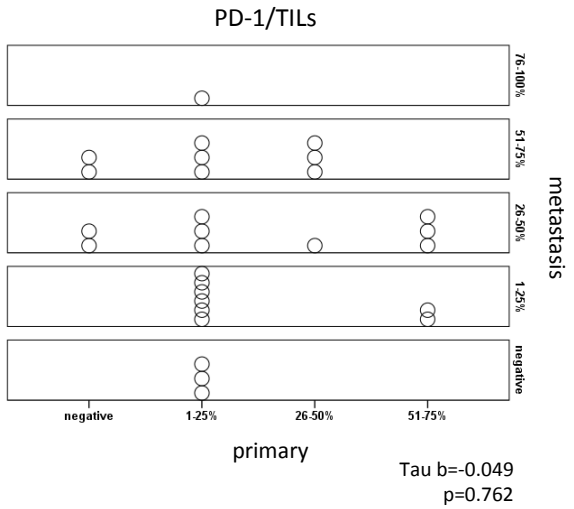
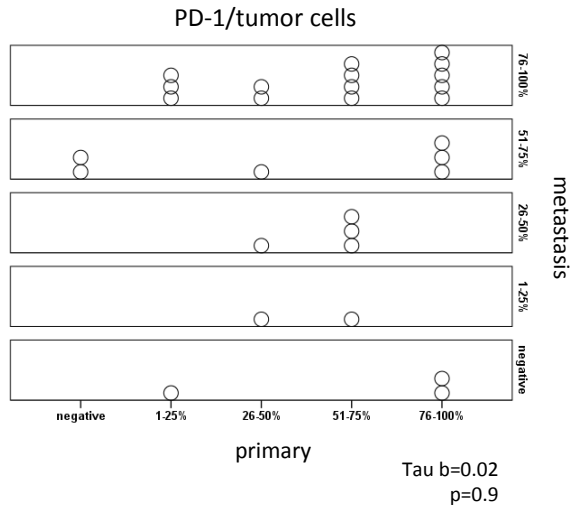

PD-1/TILs
PD-L1/TILs
negative
1-50% positive
51-100% positive
100µm

Figure 2

A



Tau b=-0.049
p=0.762



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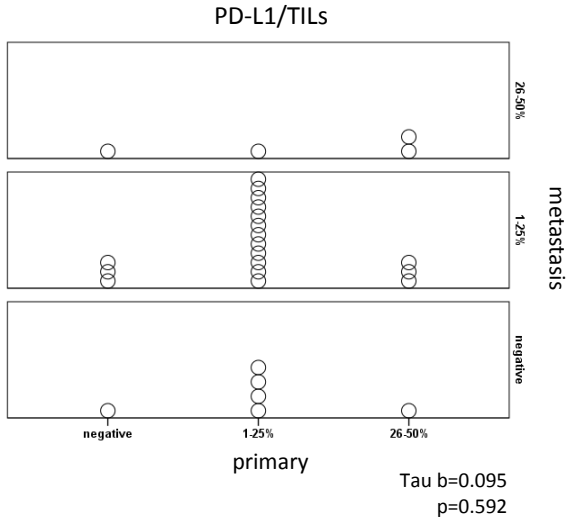
B



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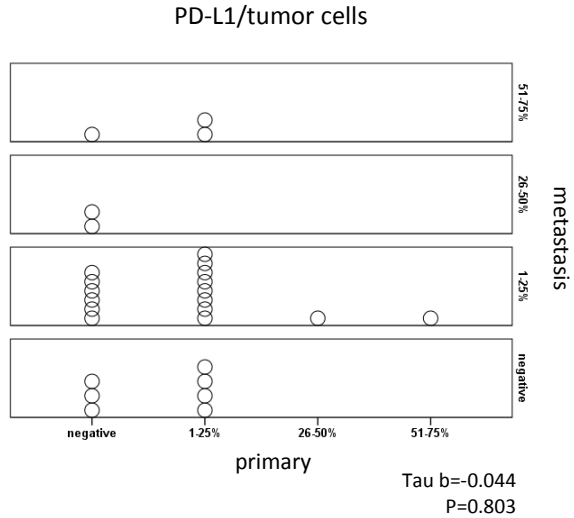

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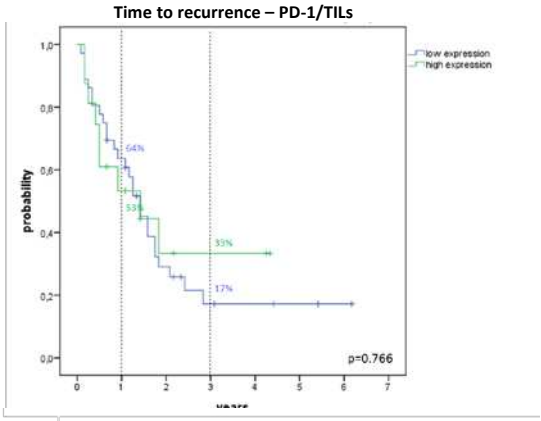
Figure 3

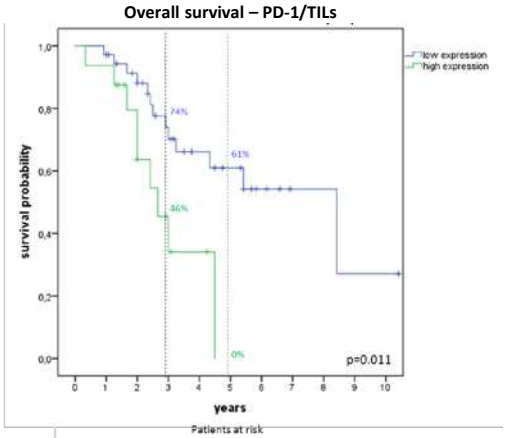
A



Patients at risk

low expression	36	22	9	4	3	2	1	0
high expression	16	7	3	2	2	0	0	0

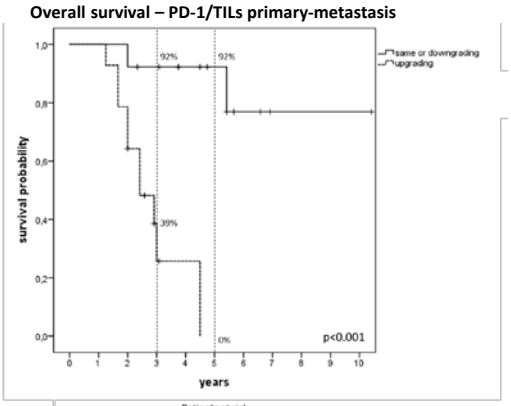
B



Patients at risk

low expression	36	35	29	20	13	10	5	2	2	1	1
high expression	16	15	10	4	2	0	0	0	0	0	0

C

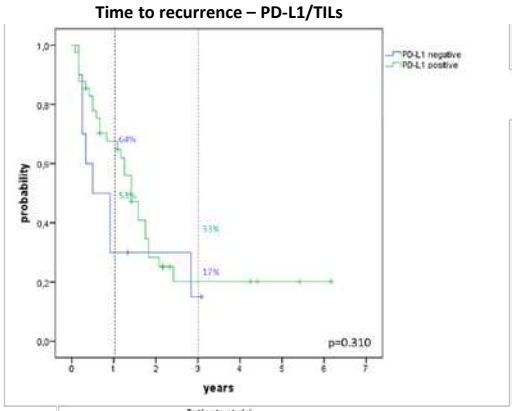


Patients at risk

same or downgrading	13	13	12	10	8	6	3	1	1	1	1
upgrading	14	14	11	3	1	0	0	0	0	0	0

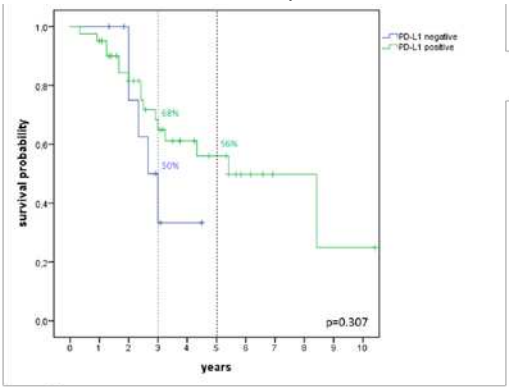
Figure 4

A



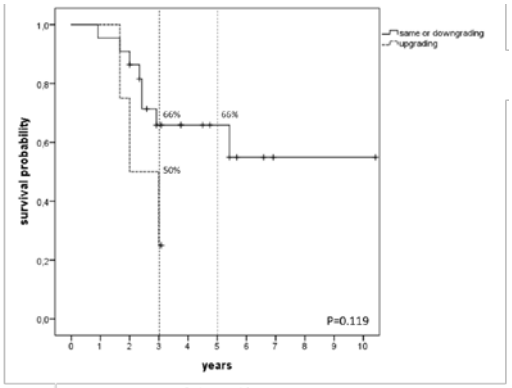
Patients at risk								
PD-L1 negative	10	3	1	0	0	0	0	0
PD-L1 positive	41	25	9	4	4	2	1	0

B



Patients at risk											
PD-L1 nevative	10	10	8	3	1	0	0	0	0	0	0
PD-L1 positive	41	39	30	20	13	10	5	2	2	1	1

C



Patients at risk											
same or downgrading	22	21	20	11	8	6	3	1	1	1	1
upgrading	4	4	3	2	0	0	0	0	0	0	0